APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1976, p. 544-550
Vol. 31, No. 4

Printed in U.S.A.

Effect of Iron on the Biodegradation of Petroleum in Seawater[1]

J. T. DIBBLE AND R. BARTHA*

Department of Biochemistry and Microbiology, Cook College, Rutgers—The State University of New Jersey, New Brunswick, New Jersey 08903

Received for publication 25 November 1975

The biodegradation of South Louisiana (SL) crude oil and the effects of nitrogen, phosphorus, and iron supplements on this process were compared in a polluted (10,900 oil degraders per liter) and in a relatively clean (750 oil degraders per liter) littoral seawater sample taken along the New Jersey coast. Without supplements, the biodegradation of SL crude oil was negligible in both seawater samples. Addition of nitrogen and phosphorus allowed very rapid biodegradation (72% in 3 days) in polluted seawater. Total iron in this seawater sample was high (5.2 μM), and the addition of iron did not increase the biodegradation rate further. In the less polluted and less iron-rich (1.2 μM) seawater sample, biodegradation of SL crude oil was considerably slower (21% in 3 days) and the addition of chelated iron had a stimulating effect. Ferric octoate was shown to have a similar stimulating effect on SL crude oil biodegradation as chelated iron. Ferric octoate, in combination with paraffinized urea and octylphosphate, is suitable for treatment of floating oil slicks. We conclude that spills of SL crude and similar oils can be cleaned up rapidly and efficiently by stimulated biodegradation, provided the water temperatures are favorable.

Although iron is one of the most abundant elements in the lithosphere (13), its precipitation as ferric hydroxide at alkaline reaction often causes this essential mineral nutrient to limit the biological productivity (23) in surface seawater to a similar degree as do nitrogen and phosphorus (24). Petroleum is a suitable source of carbon and energy for various marine microorganisms (14), but is deficient in N and P (15). The amount of metabolically available iron in crude oil is unknown. It has been demonstrated that the biodegradation of polluting petroleum in seawater is greatly stimulated by nitrogen and phosphorus supplements (3). Oleophilic forms of these nutrients were shown to promote the biodegradation of free-floating oil slicks and were proposed for use in the cleanup of oil spills untractable by conventional techniques (6). In an effort to further improve the performance of the oleophilic fertilizer formulation (a combination of paraffinized urea and octylphosphate) developed in this laboratory, the effect of iron, added either in the water-soluble or in oleophilic form, on the biodegradation of petroleum in polluted and in relatively unpolluted littoral seawater was tested.

[1] Journal paper of the New Jersey Agricultural Experiment Station, Rutgers—The State University of New Jersey, New Brunswick, N. J. 08903.

MATERIALS AND METHODS

Collection and analysis of seawater. Seawater samples were collected during the month of July, 1975 in polyethylene containers at 1-m depth, 100 m off the seaward shores of Sandy Hook State Park, N.J., and Island Beach State Park, N.J., respectively. The former location is at the southern outer edge of Raritan Bay and is fairly exposed to hydrocarbon pollutants from the Raritan Bay, the Arthur Kill, and from the New York Bay (16). Island Beach State Park is located 60 km southward from Sandy Hook and is a relatively unpolluted area. The seawater samples were used within 6 to 8 h of their collection for analysis and biodegradation experiments.

Counts of hydrocarbon-degrading microorganisms were performed on membrane filters (Millipore Corp.) incubated on the surface of modified Bushnell-Haas (Difco) agar plates, containing 1% South Louisiana (SL) crude oil as carbon source (7). The counts were performed in triplicate and were corrected for the numbers of colonies that grew in the absence of crude oil on the same mineral agar plates. The salinity of the seawater samples was measured with a YSI model 33 salinometer. Total iron in seawater was determined spectrophotometrically as described by Strickland and Parsons (22).

Biodegradation experiments. One-hundred-milliliter quantities of fresh seawater and 1 ml (0.79 g) of SL crude oil were added to 250-ml Erlenmeyer flasks. Nonbiological losses (weathering) were determined in a series of control flasks containing 1%

(wt/vol) $HgCl_2$. Water-soluble nitrogen and phosphorus were supplied as KNO_3 (10 mM) and KH_2PO_4 (0.7 mM), respectively. Oleophilic sources of N and P (6) were supplied in corresponding molar concentrations as 52.24 mg of paraffinized urea (CRNF, gift of the Sun Oil Co., Marcus Hook, Pa.) and 15.1 mg of octylphosphate (pyrophosphoric acid dioctyl ester, a gift of the Stauffer Chemical Co., New York) per 100 ml of seawater. Water-soluble iron was added as 80 μg of ferric ammonium citrate (brown, 17.5% Fe, Malinkrodt) yielding 25 μM Fe. As oleophilic sources of iron, either 120 μg of ferric octoate (ferric [2-ethylhexanoate]$_3$, a gift of the Shephard Chemical Co., Cincinnati, Ohio) or 46.5 μg of ferrocene (dicyclopentadienyliron, Aldrich) was added per 100 ml of seawater (25 μM Fe). The flasks were incubated at 28 C on a rotary shaker (150 rpm). All treatments and analyses were performed in duplicate.

Petroleum and residue analysis. SL crude oil (American Petroleum Institute reference oil sample) was obtained from J. W. Anderson, Texas A & M University, College Station, Tex. SL crude is a light, low sulfur (0.25% by weight) naphthenic-paraffinic petroleum. Its composition according to major classes of hydrocarbons (Table 1) was calculated from analytical data by Pancirov (19).

Residual oil from incubated seawater samples was recovered by four consecutive extractions with 25-ml portions of *n*-hexane. The extracts were combined and concentrated by evaporation on steam to about 25 ml. The concentrate was dried with anhydrous Na_2SO_4. The liquid phase was decanted. The solids were washed six times with small amounts of hexane and the anhydrous concentrate was adjusted with *n*-hexane to 100 ml. Quantitative analysis was performed by gas chromatography by using a Hewlett-Packard model 5700 A gas chromatograph with dual flame ionization detectors and on-column injection of 5-μl samples. The stainless-steel columns (1.8 by 3 mm) were packed with 10% Apiezon L on 60/80 mesh Chromosorb W. Operating conditions were as follows: nitrogen carrier, 30 ml/min; hydrogen, 30 ml/min; air, 240 ml/min. The detector was kept at 350 C. The oven temperature was held after sample injection for 2 min at 110 C and was subsequently programmed at a rate of 4 C/min to 250 C and held for 9 min. Under these conditions, petroleum components with equivalent carbon chain length from C_{11} to C_{25} were recorded on the chromatogram. The chromatograms were quantitated by integration of the total area response from C_{12} to C_{23}. Percentage of biodegradation was calculated by comparing the area response of biodegraded samples, after correction for nonbiological losses, to that of an unexposed oil sample. The weight losses of 10-g SL crude oil samples under a nitrogen stream and a temperature program cycle similar to that used in the gas chromatographic analysis indicated that a 17% by weight residual portion of the fresh SL crude oil was not measured by our routine gas chromatographic analysis procedure. As this proportion is not likely to stay constant during biodegradation, our data based on gas chromatographic analysis are presented without this correction. Peaks corresponding to individual *n*-paraffins were identified by their retention times as compared with authentic standards (Analabs Inc., North Haven, Conn.) and with the profile of a previously investigated Sweden (Texas) crude oil sample (4).

TABLE 1. *Composition of SL crude oil*[a]

Hydrocarbon class	Abundance (%)
Paraffins	28.0
Cycloparaffins	44.8
Aromatics	18.6
Polar compounds	8.4
Insolubles	0.2

[a] Calculated from analytical data by Pancirov (19).

Iron in SL crude oil was determined by dry ashing of 10-g samples in a muffle furnace at 850 C. The ash was dissolved in concentrated HCl and the acid was evaporated on steam. The residue was dissolved in 10 ml of 0.1 M HCl and iron was determined by atomic absorption spectrometry using a Perkin-Elmer model 290 instrument (2).

RESULTS AND DISCUSSION

Analysis of the seawater samples. The abundance of petroleum-degrading microorganisms, salinity, and total iron concentration of seawater from the two sampling sites are compared in Table 2. The higher numbers of hydrocarbon degraders at the Sandy Hook site are consistent with its proximity to known pollution sources. During the summer of 1971, the numbers of hydrocarbon degraders in the Sandy Hook area were found to be 1 order of magnitude lower than in the present study (7), but these samples were taken several kilometers off the shore. The proximity of the sampling sites to the shore in the present study readily explains the higher numbers, since wave action in shallow water results in a larger amount of suspended solids and associated microorganisms.

In consequence of the freshwater inflow from the Raritan Bay, the salinity at Sandy Hook was lower than at Island Beach. As expected, total iron was higher at the more polluted Sandy Hook site. Typically, pelagic surface seawater contains iron in true solution at 2 μg/liter (0.036 μM). Total iron is an order of magnitude higher. Due to colloidal clay particles of terrestrial origin, total iron in littoral seawater tends to be much higher and more variable (11). The iron concentrations reported in Table 2 are consistent with these accepted facts.

Biodegradation of SL crude oil in polluted and unpolluted seawater. When corrected for nonbiological losses (10% in 15 days), biodegra-

TABLE 2. *Petroleum-degrading microorganisms, salinity, and total iron in littoral seawater off the New Jersey coast*[a]

Site	Colony-forming units/liter	Standard deviation	Salinity (‰)	Total iron (μM)	Standard deviation
Sandy Hook	10,900	1,330	19	5.4	0.32
Island Beach	750	160	30	1.2	0.15

[a] Samples taken 100 m off the shores of Sandy Hook and Island Beach State Parks, respectively. At the time of sampling (July, 1975), the water temperature at both locations was 20 C. The presence of suspended sediment, especially at Sandy Hook, may have resulted in low colony counts. The figures listed here should be regarded as minimal rather than absolute.

dation of SL crude oil in unsupplemented seawater from both collection points was insignificant (Fig. 1). When nitrate and phosphate were added at levels found to be optimal in earlier studies on Sweden crude oil (3), biodegradation was very rapid in the more polluted Sandy Hook water and occurred at a fair rate in the less polluted Island Beach water. This effect of nitrogen and phosphorus on oil biodegradation in seawater was noted earlier in this (3) as well as in other laboratories (10, 11, 21), but some quantitative differences are noteworthy. Biodegradation in nitrate- and phosphate-enriched seawater was remarkably rapid as compared to the biodegradation of Sweden crude oil tested under identical conditions (3). Sweden crude oil was shown to have volatile components that are bacteriostatic (5) and cause a temperature-dependent lag period in biodegradation. Maximal biodegradation of Sweden crude oil (~70%) at 28 C in Sandy Hook water required 3 to 4 weeks (3). Oil spill cleanup by stimulated biodegradation was judged to be of value where physical cleanup is technically impossible, but the slowness of the process was considered to be a major drawback (8). In the present tests with SL crude oil, comparable biodegradation (73%) was achieved in 3 days using Sandy Hook water. It is evident from Fig. 2 that, besides the normal alkane peaks, most of the unresolved "envelope" of branched alkanes, cycloalkanes, and aromatics was also degraded. In the less polluted Island Beach water the biodegradation process was slower and less complete (see Fig. 5B).

SL crude oil is apparently substantially less bacteriostatic than the previously tested Sweden crude oil. It is encouraging to note that the biodegradation of SL crude oil, which is representative of much of the U.S. Gulf Coast production, can attain a rate and extent that compares favorably with much more costly cleanup methods. Admittedly, this occurs under optimal conditions, but a temperature of 28 C is not unrealistic for the shallow waters of the Gulf Coast, and nitrogen and phosphorus can be supplied in oleophilic form to free-floating slicks (6).

An earlier survey of the numbers of hydrocarbon degraders and the oil biodegradation potential in the Raritan Bay showed an inverse correlation between the former numbers and the distance to known pollution sources, but no correlation with the biodegradation potential was discerned (7). A more significant difference between the sampling sites, a closer monitoring of the time course of the biodegradation and the less inhibitory nature of the SL crude oil were probably all helpful in revealing such a correlation in the present study.

Effect of chelated iron on the biodegradation of SL crude oil in polluted and unpolluted seawater. The specific levels of iron required by hydrocarbon-degrading bacteria are not available from the literature. The minimum iron concentration that allowed the attainment of a 1.28-mg/ml protein yield (approximately 2.1 mg/ml [dry weight]) by the facultatively chemolithotrophic bacterium *Alcaligenes eutrophus* strain H1 (formerly *Hydrogenomonas* strain H1) is 2.5 μM or 14 μg of Fe per 100 ml of medium (9). Determination of iron in SL crude oil by atomic absorption spectrometry yielded 7.2 ppm (wt/wt) or 5.7 μg/ml. Centrifugation of SL crude oil at 10,000 rpm for 15 min before iron determination did not lower this value.

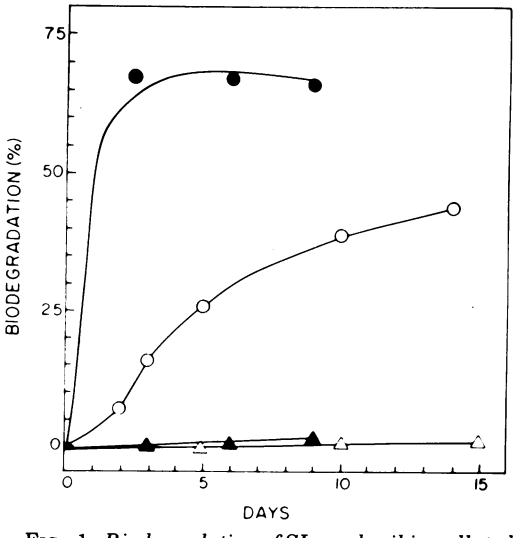

FIG. 1. *Biodegradation of SL crude oil in polluted Sandy Hook seawater (solid symbols) and relatively clean Island Beach seawater (hollow symbols). Symbols: Triangles, unsupplemented seawater; circles, nitrate and phosphate was added.*

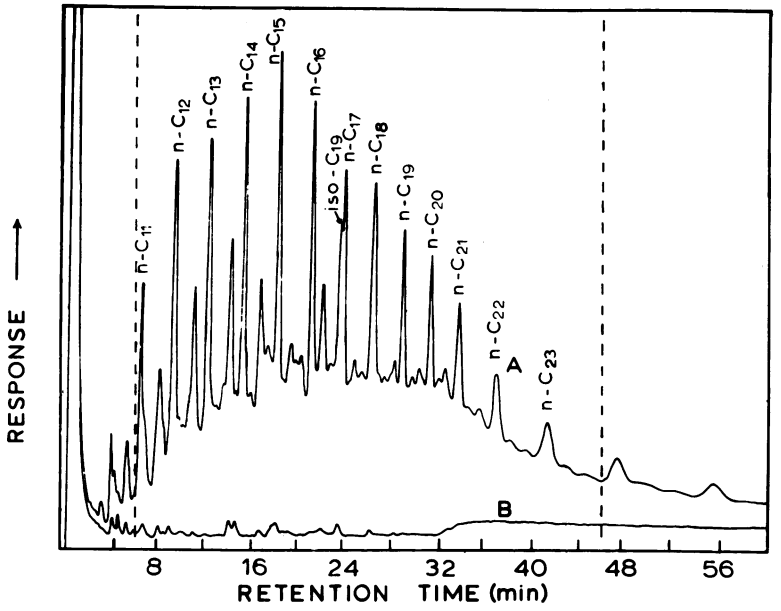

FIG. 2. *Gas chromatograms of SL crude oil incubated for 3 days in (A) unsupplemented and (B) nitrate- and phosphate-enriched polluted Sandy Hook seawater. The integrated portions of the chromatograms are enclosed in brackets. Tracing A is undistinguishable from that of undegraded SL crude oil (not shown). The major alkane peaks are identified.*

Consequently, the determined iron was regarded to be organometallic rather than mineral. The metabolic availability of this iron being unknown, a contribution from this source towards the production of cell biomass was not assumed in the subsequent calculations. Depending on the seawater sample (see Table 2), the total iron in a 100-ml amount was calculated to be 6.7 and 30.0 μg, respectively. Most of this iron is not in solution but associated with particles (11) and is not available for immediate incorporation by microorganisms. Hydrocarbons can be converted to dry cell material with up to 100% efficiency (12), and in this manner the biodegradation of 70% of 1 ml of SL crude oil could yield up to 550 mg of cell dry weight. Assuming that the iron requirement per cell dry weight of *A. eutrophus* strain H1 and the hydrocarbon degraders is similar, the discrepancy between the required (26.7 μg) and the actually available iron, especially in the less polluted seawater sample, could well limit the total cell yield and thus the rate of oil biodegradation. As the specific iron requirement of hydrocarbon degraders was not determined in these studies, iron was added at 25 μM concentration, i.e., at the 10-fold level that permitted a 2.1-mg/ml cell yield of *A. eutrophus* strain H1. This was done to insure that both a higher cell yield and a possibly higher specific iron demand would be satisfied by the added iron.

Figure 3 shows the effect of chelated iron on the biodegradation rate of SL crude oil in Sandy Hook and Island Beach water. In the more polluted and iron-rich water sample, the stimulation of the biodegradation rate was marginal. In the less polluted and less iron-rich water sample there was a clear stimulation of the biodegradation rate by added iron, though the effect was far less dramatic than that of nitrogen and phosphorus. The experiment suggests that iron, along with nitrogen and phosphorus, may limit petroleum biodegradation to some degree in relatively clean coastal waters, and even more so in pelagic seawater. Iron is not likely to limit petroleum biodegradation in coastal waters that receive substantial amounts of terrigeneous sediments. The temporary nature of the limitation in the less iron-rich seawater sample suggests that in the course of extensive petroleum biodegradation some of the organometallic iron may become available for the degrading microorganisms, but utilization of intracellular iron in the course of population turnover furnishes an equally plausible explanation.

Effect of oil-soluble iron in combination with oleophilic nitrogen and phosphorus on the biodegradation of SL crude oil. The following experiments were conducted with Island

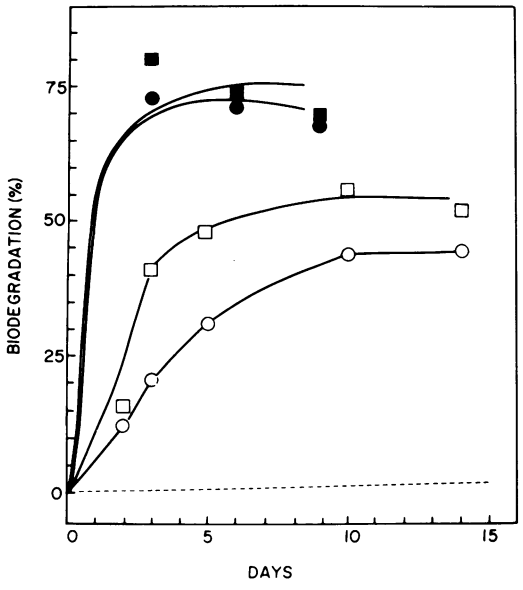

FIG. 3. *Effect of chelated iron on the biodegradation of SL crude oil in polluted Sandy Hook seawater (solid symbols) and relatively clean Island Beach seawater (hollow symbols). Symbols: Circles, only nitrate and phosphate were added; squares, nitrate, phosphate plus chelated iron were added. The dashed line shows, for comparison, biodegradation in unsupplemented Island Beach seawater (see Fig. 1).*

Beach water only, since an iron limitation was not evident in the Sandy Hook water sample. For the reasons discussed in an earlier publication (6), the stimulated biodegradation of a floating oil slick requires oil-soluble rather than water-soluble sources of the required mineral nutrients. From the available organometallic iron compounds, on the basis of their solubility characteristics, ferrocene and ferric octoate were selected for use in biodegradation tests. Ferrocene performed poorly in these tests, apparently because of biological recalcitrance. Ferric octoate, an oily substance with a strong reddish-brown color, failed to stain the water phase but dissolved homogeneously in SL crude oil. It stimulated its biodegradation to a similar degree as did chelated iron (Fig. 4). In combination with paraffinized urea and octylphosphate, it is judged to be of use as oleophilic fertilizer for stimulated biodegradation of floating oil slicks. Ferric octoate is quoted by the manufacturer at $2.20/kg. At our projected treatment levels, 1 metric ton of crude oil requires 150 g of this material. Hence, the incorporation of ferric octoate would add only minimally to the estimated cost ($15.00/metric ton of oil) of the oleophilic nitrogen and phosphorus (6). Figure 5 shows the gas chromatographic tracings obtained after 3 days of biodegradation. At this early time in unsupplemented seawater (A), no change is evident. Sample (B), supplemented with nitrogen and phosphorus only, biodegradation is largely restricted to *n*-alkane peaks, while the addition of iron (C) substantially reduces also the size of the unresolved "envelope." Pristane (2,6,10,14-tetramethylpentadecane, labeled as iso-C_{19} in Fig. 5) is relatively resistant to biodegradation as repeatedly observed before (4, 18), but it is also obvious that some biodegradation of this compound had occurred before the *n*-alkanes were exhausted. The diauxic phenomenon described for pure cultures of *Brevibacterium erythrogenes* (20) does not necessarily apply to mixed microbial communities, and the use of pristane as "internal standard" in such cases (18) may yield erroneous results.

Figures 4 and 5, in comparison to Fig. 1 and 2, also show that in the less polluted Island Beach water biodegradation, even at 15 days, does not equal the biodegradation in Sandy Hook water at 3 days. In less polluted marine environments, the augmentation of the natural biodegradation potential by inoculation with suitable hydrocarbon-degrading microorganisms (1) seems appropriate as long as it is combined with adequate fertilization. A recent report (D. A. Friello, J. R. Mylroie, and A. M. Chackrabarty, Third Int. Biodegradation

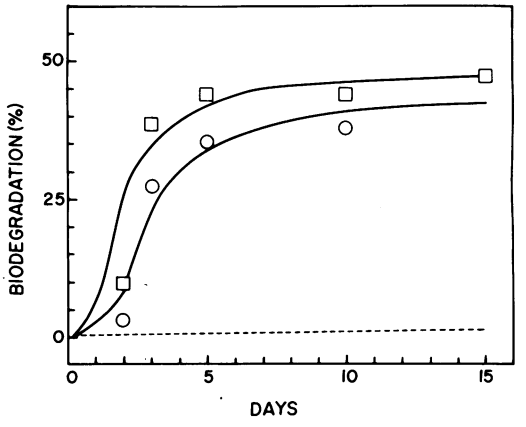

FIG. 4. *Biodegradation of SL crude oil in relatively unpolluted Island Beach seawater, as stimulated by oleophilic fertilizers. Symbols: Circles, paraffinized urea and octylphosphate were added; squares, paraffinized urea, octylphosphate and iron octoate were added. The dashed line shows, for comparison, biodegradation in unsupplemented seawater (see Fig. 1).*

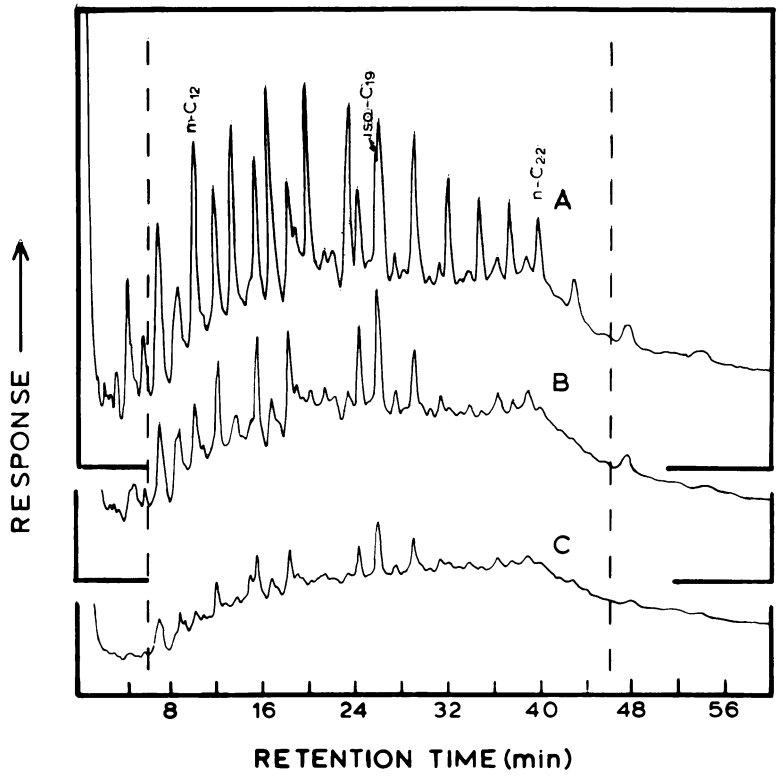

FIG. 5. *Gas chromatograms of SL crude oil incubated for 3 days in relatively unpolluted Island Beach seawater. (A) Unsupplemented seawater; (B) oleophilic nitrogen and phosphorus were added; (C) oleophilic nitrogen, phosphorus, and iron were added. Tracing A is undistinguishable from that of undegraded SL crude oil (not shown). The integrated portions of the chromatograms are enclosed in brackets. Some major alkane peaks are identified.*

Symp., Aug. 17-23, Kingston, R.I., Abstr. 17, p. 7, 1975) on the expansion of the substrate range of a hydrocarbon-degrading *Pseudomonas putida* strain is an encouraging step towards the development of highly effective microbial inocula for oil cleanup.

ACKNOWLEDGMENTS

This work was supported by the Office of Naval Research and by New Jersey State Funds. We gratefully acknowledge the cooperation of A. Markus in the performance of the iron analysis on SL crude oil.

LITERATURE CITED

1. **Anonymous.** 1970. Microorganisms consume oil in test spills. Chem. Eng. News 48:48-49.
2. **Anonymous.** 1971. Analytical methods for atomic adsorption spectrophotometry (instrument manual) petroleum, PC5. Perkin Elmer Corp., Norwalk, Conn.
3. **Atlas, R. M., and R. Bartha.** 1972. Degradation and mineralization of petroleum in seawater: limitation by nitrogen and phosphorus. Biotechnol. Bioeng. 14:309-317.
4. **Atlas, R. M., and R. Bartha.** 1972. Degradation and minieralization of petroleum by two bacteria isolated from coastal waters. Biotechnol. Bioeng. 14:297-308.
5. **Atlas, R. M., and R. Bartha.** 1972. Biodegradation of petroleum in seawater at low temperatures. Can. J. Microbiol. 18:1851-1855.
6. **Atlas, R. M., and R. Bartha.** 1973. Stimulated biodegradation of oil slicks using oleophilic fertilizers. Environ. Sci. Technol. 7:538-541.
7. **Atlas, R. M., and R. Bartha.** 1973. Abundance, distribution and oil biodegradation potential of microorganisms in Raritan Bay. Environ. Pollut. 4:291-300.
8. **Bartha, R., and R. M. Atlas.** 1972. Stimulated biodegradation of polluting oil at sea. Oceanic Abstr. 9:4-7.
9. **Bartha, R., and E. J. Ordal.** 1965. Nickel-dependent chemolithotrophic growth of two *Hydrogenomonas* strains. J. Bacteriol. 89:1015-1019.
10. **Bridie, A. L., and J. Bos.** 1971. Biological degradation of mineral oil in sea water. J. Inst. Pet. 57:270-277.
11. **Cooper, L. H. N.** 1935. Iron in the sea and in marine plankton. Proc. R. Soc. London Ser. B 118:419-438.
12. **Coty, V. F., and R. I. Levitt.** 1971. Microbial protein from hydrocarbons. Dev. Ind. Microbiol. 12:61-71.
13. **Deevey, E. S., Jr.** 1970. Mineral cycles. Sci. Am. 223:149-158.
14. **Friede, J., P. Guire, R. K. Gholson, E. Gaudy, and A. F. Gaudy, Jr.** 1972. Assessment of biodegradation potential for controlling oil spills on the high seas. United States Coast Guard report no. 4110.113.1. U.S. Government Printing Office, Washington, D.C.
15. **Hunt, R. H., and M. J. O'Neal.** 1967. Petroleum (composition), p. 845-855. *In* R. E. Kirk and D. F. Othmer,

Encyclopedia of chemical technology, vol. 14, 2nd ed. Wiley Interscience, New York.
16. **Jeffries, H. P.** 1962. Environmental characteristics of Raritan Bay, a polluted estuary. Limnol. Oceanogr. 7:21-31.
17. **Kator, H. I., R. Miget, and C. H. Oppenheimer.** 1972. Utilization of paraffin hydrocarbons in crude oil by mixed cultures of marine bacteria. *In* Symposium on environmental conservation, Nov. 13-14, 1972, Lafayette, La., paper no. SPE 4206. AIME, Dallas, Tex.
18. **Miget, R. J., C. H. Oppenheimer, H. I. Kator, and P. A. LaRock.** 1969. Microbial degradation of normal paraffin hydrocarbons in crude oil, p. 327-331. *In* Proceedings, prevention and control of oil spills, API, FWPCA Dec. 15-17, Washington, D.C. American Petroleum Institute, New York.
19. **Pancirov, R. J.** 1974. Compositional data on API reference oils used in biological studies: a #2 fuel oil, a Bunker C, Kuwait crude oil and South Louisiana crude oil. Report no. AID. IBA. 74 Esso Research, Linden, N.J.
20. **Pirnik, M. P., R. M. Atlas, and R. Bartha.** 1974. Hydrocarbon metabolism of *Brevibacterium erythrogenes*: normal and branched alkanes. J. Bacteriol. 119:868-878.
21. **Reisfeld, A., E. Rosenberg, and D. Gutnick.** 1972. Microbial degradation of crude oil: factors affecting the dispersion in sea water by mixed and pure cultures. Appl. Microbiol. 24:363-368.
22. **Strickland, J. D. H., and T. R. Parsons.** 1968. A practical manual of seawater analysis, p. 97-107. Fisheries Research Board of Canada, Ottawa.
23. **Sverdrup, H. U., M. W. Johnson, and R. H. Fleming.** 1970. The oceans, p. 769. Prentice-Hall, Englewood Cliffs, N.J.
24. **Sverdrup, H. U., M. W. Johnson, and R. H. Fleming.** 1970. The oceans, p. 235-263. Prentice-Hall, Englewood Cliffs, N.J.